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The overall goal of this project is the development of conditionally-replicating adenoviruses ('CRA') that will selectively infect, replicate in, and kill prostate tumor cells, with the spread of progeny virus to further tumor cells. While CRA have been described before, all have been based on the tropism of the commonly used adenovirus type 5 (Ad5), which 1) does not efficiently infect many tumor cells, and 2) infects a wide variety of normal cells. By improving infection of the tumor cells and the selectivity of conditionally replicating viruses, we hope to improve the therapeutic window associated with replicating Ad treatment. There are three Aims (Tasks) in the project: 1) to evaluate existing Ad serotypes for their possible utility, 2) to use phage display technology to isolate peptides that selectively bind to prostate tumor cells, and 3) to combine the best targeting strategy produced in the first two aims with prostate tumor-selective Ad replication. We have made substantial progress in both of these aims and have identified two very promising fiber proteins for further use. Based on these findings, we feel that we are on track to meet the overall goal of constructing and evaluating a retargeted conditionally replicating virus.

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Introduction

The overall goal of this project is the development of adenoviruses (conditionally-replicating adenoviruses, 'CRA') that will selectively infect, replicate in, and kill prostate tumor cells, with the spread of progeny virus to further tumor cells. While CRA have been described before, all have been based on the tropism of the commonly used adenovirus type 5 (Ad5), which 1) does not efficiently infect many tumor cells, and 2) infects a wide variety of normal cells. Both of these issues are likely to reduce the efficacy of treatment and lead to increased toxicity. By improving infection of the tumor cells and the selectivity of conditionally replicating viruses, we hope to improve the therapeutic window associated with replicating Ad treatment. There are three Aims (Tasks) in the project: 1) to evaluate existing Ad serotypes for their possible utility, 2) to use phage display technology to isolate peptides that selectively bind to prostate tumor cells, and 3) to combine the best targeting strategy produced in the first two aims with prostate tumor-selective Ad replication. In years One and Two of the proposal, work was planned for only the first two Aims. As detailed below, we have made substantial progress in both of these aims and have identified two very promising fiber proteins for further use. Based on these findings, we feel that we are on track to meet the Year Three goal of constructing and evaluating a retargeted conditionally replicating virus.

Task 1: Evaluate fiber proteins encoded by the naturally occurring human adenoviruses for their ability to infect prostate tumor cells (months 1-18).

Evaluation of fiber proteins from each subgroup of human adenovirus

In Year 1, we identified the Ad16 and Ad35 fibers as having the broadest abilities to infect prostate tumor cells. We are now in the process of generating Ad5 vectors with these fiber proteins inserted in place of the Ad5 fiber (see Task 3). As an example of this strategy, a derivative of the pAdEasy plasmid with the Ad16 fiber has been constructed and successfully used to recover a GFP-marked Ad with the Ad16 fiber substituted for that of Ad5 (Figure 1). A similar strategy will be used to construct the Ad16-targeted conditionally replicating Ad (Year 3). An analogous plasmid with the Ad35 fiber substituted in place of the Ad5 fiber is under construction.

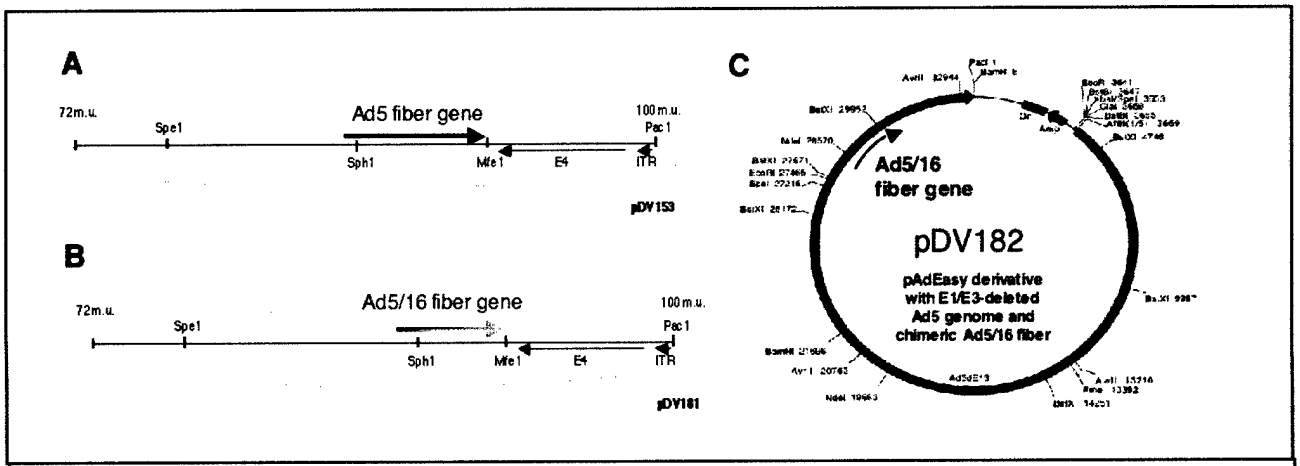

Fig. 1: Construction of plasmids for rapid generation of Ad vectors with the chimeric Ad5/Ad16 fiber gene. A) pDV153 contains Ad5 sequences from 72-100 m.u. with the unique Mun1 site relocated to be immediately downstream of the fiber ORF. This facilitates substitutions of the fiber shaft/knob domains as Sph1/Mun1 fragments. 2) pDV181 results from substitution of the Ad16 fiber shaft/knob for that of Ad5 in pDV153. C) pDV182 is a derivative of pAdEasy with the chimeric Ad5/16 fiber gene, constructed by susbstituting the Spe1/Pac1 fragmetn from pDV181 for that of pAdEasy. This plasmid is used in the generation of retargeted Ad genomes by bacterial recombination.

Task 2: Use a phage display system to identify peptides that can be used for targeting adenovirus vectors to prostate tumor cells (months 1-24)

As reported in the 2002 progress report, we recovered a number of tumor cell-binding peptides. Over the last year, we have completed evaluation of the infectivity of Ads targeted with these peptide sequences.

Three motifs (HAIYPRH, SILPYPY, and SAVHLSA) were recovered multiple times in Experiment 1. In *in vitro* assays, all three bound significantly better than unselected library phage to the PC-3 cells, and more poorly than the library phage to the HepG2 cells. We extended the in vitro binding to other prostate tumor cell lines, and found that phage expressing the HAIYPRH and SAVHLSA peptides were able to bind to DU145, LNCaP, and PC-3 cells. In experiment #2 three peptide sequences (NGYSWTS, MGTPPWR, and MILPQKV) were recovered multiple times, accounting for the majority (79%) of sequenced clones. Of these, NGYSWTS and MILPOKV proved to have the most favorable binding to the tumor cells.

Addition of the recovered peptides to a 'detargeted' fiber protein

The peptides were inserted into a genetically 'detargeted ' Ad5 fiber protein which lacks CAR binding due to the presence of a double point mutation (KO1; Jakubczak et al. 2001). We have inserted a short linker sequence and a unique restriction site for insertion of the peptide sequences into the HI loop of this fiber Nicklin et al., 2001) (**Fig. 2**).

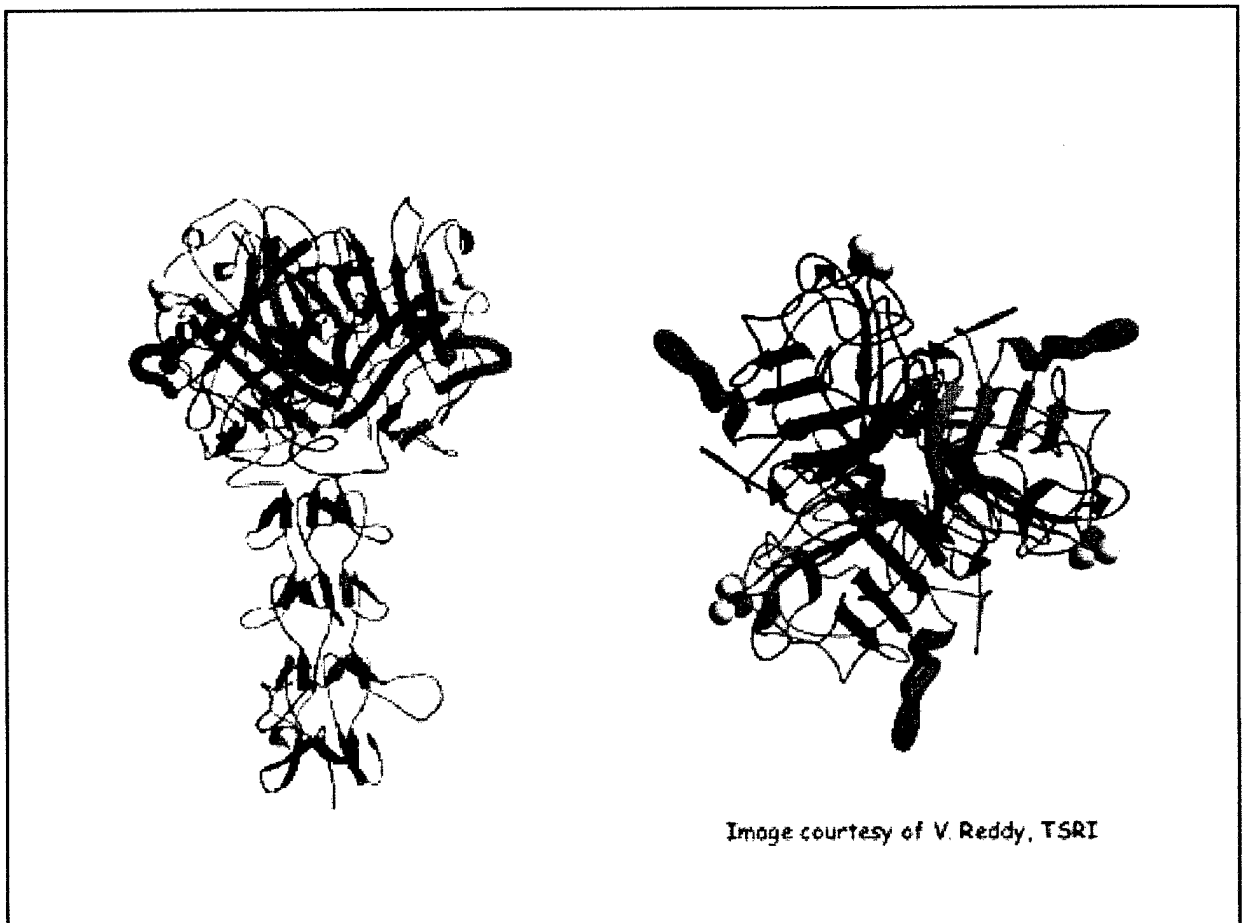

Fig. 2: Side (left) and top (right) views of the Ad5 fiber knob crystal structure. The location of the KO1 point mutations that block CAR binding are shown as blue spheres. The HI loop (site of peptide insertion) is indicated by the bold purple line.

Ad particles with the modified proteins were then generated using a rapid transfection/infection protocol. We found that all of the resulting proteins formed stable trimers and assembled onto particles at essentially normal levels (Fig. 3). These viral preparations were then tested for their ability to infect prostate tumor cells as well as non-prostate derived cells (Figs. 4 and 5).

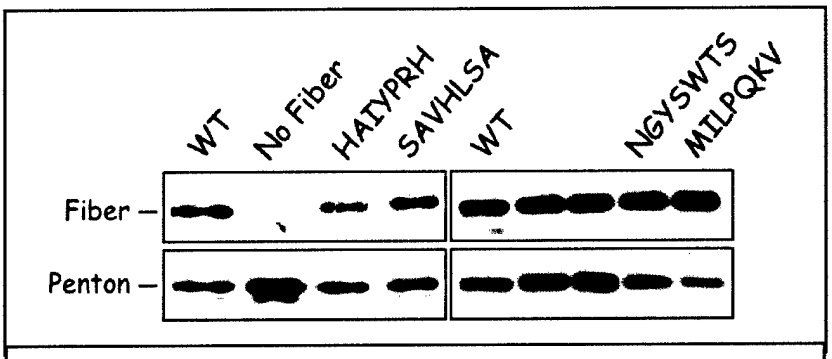

Fig. 3: Generation of Ad particles with peptide-modified fiber proteins. 293T cells were transfected with expression constructs encoding the various fiber proteins, and 24 hours later infected with the fiber-deleted Ad5 vector Ad5.GFP.ΔF. Approximately 48 hours postinfection, virus was harvested and purified by CsCl gradient ultracentrifugation. Five micrograms of each viral preparation was then subjected to Western blot analysis. Blots were probed with polyclonal antibodies against the fiber (upper panels) or penton base (loading control; lower panels) proteins. Lanes marked 'wt' contain a first-generation Ad5 vector as a standard. Lanes containing peptide-modified fibers are identified by the peptide sequence inserted into the fiber protein.

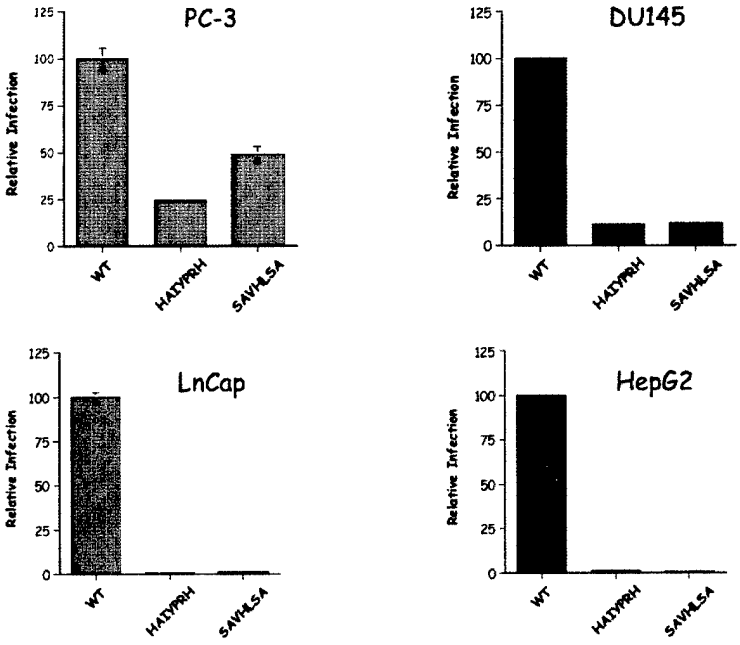

Fig. 4. Infection of prostate tumor cell lines by Ads targeted with peptides recovered in screen #1. Cells were incubated for three hours with 5000 particles/cell of Ad5.GFP.ΔF equipped with the wt) fibers, or with the KO1 fiber with HI loop insertion of HAIPRYH or SAVHLSA peptides. Twenty four hours post-infection, GFP fluorescence was assessed by FACS. Fraction of GFP-positive cells are expressed relative to the percent GFP-positive using the Ad5 fiber.

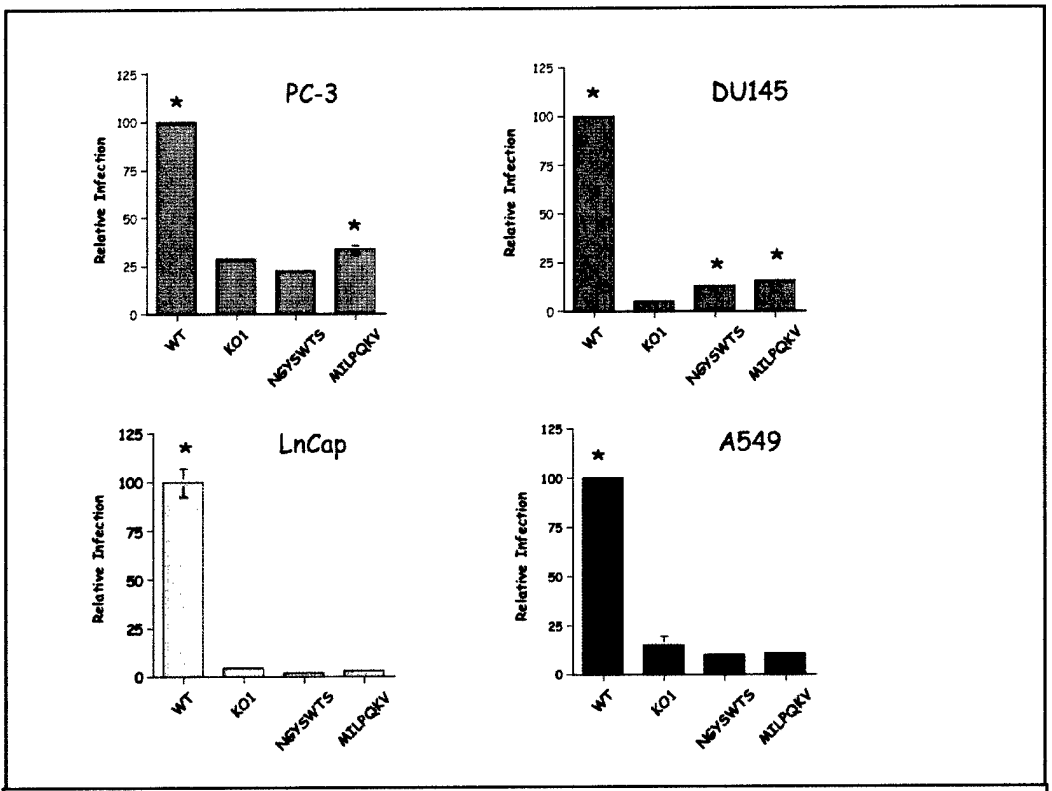

Fig. 5. Infection of prostate tumor cell lines by Ads targeted with peptides recovered in screen #2. Cells were incubated for three hours with 5000 particles/cell of Ad5.GFP.ΔF equipped with the wt or KO1 (detargeted) fibers, or with the KO1 fiber with HI loop insertion of NGYSWTS or MILPOKV peptides. Twenty four hours post-infection, GFP fluorescence was assessed by FACS. Fraction of GFP-positive cells are expressed relative to the percent GFP-positive using the Ad5 fiber. '*': $p < 0.05$ from KO1 (Student's T test).

In summary, the results from panning of the linear library have been relatively disappointing. The two best peptides evaluated from Experiment #2 (NGYSWTS and MILPQKV) did confer significantly improved infection of PC-3 and DU-145 cells relative to the detargeted fiber lacking a peptide insert (Fig. 5), but it is unclear if this level of infection will be sufficient for eventual use in a targeted vector. Additionally, SAVHLSA was able to confer increased infection on PC-3 but not other cell lines (Fig. 4), suggesting that it does not bind a receptor generally expressed on tumor cells. The phage panning experiments described were done following published standard protocols. After discussion with other workers in this field, we are now in the process of re-screening these libraries under more stringent conditions in hopes of identifying ligands with more favorable binding properties, as well as of screening a constrained library.

Task 3: Construct and test a conditionally-replicating adenovirus incorporating the most prostate tumor-specific fiber protein identified in (1 and 2) for use in anti-tumor therapy (months 25-36)

As this Task is dependent on identification of the most tumor-selective fiber protein in Tasks 1 and 2, no work was planned for year two of the funding period. As noted above, we have identified the Ad16 and Ad35 fibers as the most promising natural fibers and are in the process of constructing and evaluating reporter gene-marked Ads with these fibers in substituted for that

of Ad5. The plasmids used for construction of these viruses will also be used in the final phase of the project in construction of the conditionally-replicating viruses. We are also re-screening the phage libraries with the goal of isolating peptides with better binding properties, which would then be incorporated into virus using the same strategy.

Key Research Accomplishments

Addition of tumor cell-binding peptides to the HI loop of the Ad5 fiber.

Generation and evaluation of viruses containing the modified fiber proteins.

Construction of plasmids for use in producing prostate tumor-specific replicating Ads targeted with the Ad16 and Ad35 fibers.

Reportable Outcomes

The viruses with the Ad16 or Ad35 fibers substituted into the chromosomes are valuable reagents for studies of the tropism of these Ad serotypes in general as well as in the work covered under this proposal.

Conclusions

Progress has been made on evaluation of both targeting strategies. We have identified two native fiber proteins (Ad16 and Ad35) that infect a panel of prostate tumor cell lines much more effectively than do the standard Ad5 vectors, and are now constructing viruses with these fibers substituted for that of Ad5 in the viral chromosome. Our phage display work has not yet yielded peptide targeting that appears better than what can be achieved using native fibers, although we plan to revisit this strategy before abandoning it altogether. Overall, we feel we are in an excellent position to construct and test replicating Ads with alternate receptor targeting in the next year, as planned. This should in turn lead to viruses suitable for testing in pre-clinical models.

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